Appendix A

QUALITY ASSURANCE STATEMENT

The Quality Assurance Unit (QAU) has inspected the conduct of different phases of the study according to a predetermined testing schedule. To the best of our knowledge, there were no deviations from the protocol and standard operating procedures that would affect the integrity of this study.

This report has been audited by the QAU in accordance with the appropriate standard operating procedures of the Drug Development Division, DCB. The report is considered to describe the methods and procedures used in the study, and the reported results accurately reflect the raw data generated during this study.

Listed below are the phases in this study that were audited by the QAU and the dates the audits were performed and findings reported to management.

Audit Date	Phase Audited	Date Reported to Study Director	Date Reported to Management
Nov. 14, 2001	Protocol	Nov. 14, 2001	Nov. 14, 2001
Nov. 20, 2001	Test article treatment (-S9)	Nov. 22, 2001	Nov. 22, 2001
Nov. 21, 2001	Cytotoxicity test and expression of the mutant phenotype	Nov. 22, 2001	Nov. 22, 2001
Nov. 22, 2001	Test article treatment (+S9)	Nov. 23, 2001	Nov. 26, 2001
Dec, 26, 2001	Raw data; study records	Dec, 28, 2001	----------
Jan. 14, 2002	Final report	Jan. 15, 2002	Jan. 15, 2002

Wen-Jing Chen — Jan 15, 2002

Wen-Jing Chen
Quality Assurance Officer

Date

TESTING FACILITY

Name: Genetic Toxicology Laboratory, Drug Development Division, Development Center for Biotechnology

Address: 103, Lane 169, Kang-Ning St., Hsi-Chih City, Taipei County 221, Taiwan, R.O.C.

SPONSOR

Name: MICROBIO Co., Ltd.

Address: No. 81, Gauyang N. Road, Lung Tan Shiang, Tao Yuan, Taiwan, R.O.C.

Representative: William Lu

TEST ARTICLE (Information was supplied by the sponsor prior to study initiation)

A. Name/Identification: Product code MicrSoy-20 (MS-20)

B. Receiving Date: Oct. 05, 2001

C. Batch/Lot Number: 20010209

D. DCB Code: DV00199-1

E. Ingredients: The components are very complicated. Until now, its effective components are still unable to determine.

F. Storage Conditions: Room temperature and protect from light

G. Expiration Date: Feb. 09, 2004

H. Physical Appearance: Dark-brown liquid with prune juice odor

Statements:

1. **The test article is a proprietary product of the sponsor, therefore the sponsor will be responsible for the requirements listed under "Test Article" of the GLP regulation (21CFR § 58.105, FDA).**
2. **The testing result is effective for submitted sample only, and shall not be excerpted from the contents of this report without the written approval of the testing facility.**
3. **The testing result and report are generated by DCB for the test article submitted by the sponsor, and are intended for petition to government agency for product registration.**

TEST SCHEDULE

A. Date of test article treatment in the absence of S9 activation: Nov. 20, 2001

B. Date of test article treatment in the presence of S9 activation: Nov. 22, 2001

C. Date of completion of colony counting: Dec. 10, 2001

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Appendix B

Protocol

HPRT GENE MUTATION ASSAY
PRODUCT CODE MICRSOY-20 (MS-20)

SUMMARY

The test article, Product code MicrSoy-20 (MS-20) supplied by MICROBIO Co., Ltd. was studied with HPRT gene mutation assay in Chinese hamster ovary cells (CHO/HPRT) in the absence and presence of Aroclor 1254-induced rat liver S9. A preliminary cytotoxicity assay was used to establish the concentration range for the mutagenesis assay. The definite mutagenesis assay was used to evaluate the mutagenic potential of the test article.

Test article stock solution was prepared in cell culture medium and filtered through a 0.2 μm membrane after adjusting pH value to the range of 7.0 ~ 7.2. Cell culture medium was used to further dilute test article solutions to desired concentrations for cell treatment. Selection of concentration levels for the mutagenesis assay was based on the solubility and cytotoxicity in the test system. The cytotoxicity of Product code MicrSoy-20 (MS-20) was determined by the colony formation test. According to the test results, the top concentration was set as 100 μl/ml for the absence of S9 activation and 50 μl/ml for the presence of S9 activation in the gene mutation assay.

The HPRT gene mutation assay was analyzed with 6 different concentrations, 3.125, 6.25, 12.5, 25, 50 and 100 μl/ml for test without S9 activation and 1.5625, 3.125, 6.25, 12.5, 25 and 50 μl/ml for test with S9 activation for 5-hour MS-20 treatment. All the tests were conducted in duplicate cultures and with solvent control and positive controls concurrently. The cells were analyzed for cytotoxicity after drug treatment. Allowing a period of phenotype expression, the treated cultures were selected for 6-TG resistant colonies. The mutation frequency was calculated based on 10^6 clonable cells. The concurrent cytotoxicity assays showed 83.2% and 80.8% toxicity (cloning efficiency relative to the solvent control) at 50 μl/ml in the absence and presence of S9, respectively. The results of negative and positive controls showed that all the criteria for a valid test were met. In the mutagenesis assay, none of test concentration either with or without S9 activation had induced more than 40 mutants per 10^6 clonable cells, i.e. no positive responses.

Under the conditions of this study, test article Product code MicrSoy-20 (MS-20) was concluded to be negative in the CHO/HPRT gene mutation assay.

INTRODUCTION

The objective of this study was to evaluate the mutagenic potential of test article based on quantitation of forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus of Chinese hamster ovary cells.

MATERIALS AND METHODS

A. Test System

Chinese hamster ovary cells (CHO-K1) were obtained from American Type Culture Collection (ATCC, repository number CCL-61), Rockville, MD, USA. The CHO-K1 cell line has an epithelial-like morphology and a modal chromosome number of 20. CHO cells were cleansed in medium supplemented with hypoxanthine, aminopterin and thymidine (HAT) to reduce the background mutation rate and frozen. The frozen lot of cells was tested and found to be free of mycoplasma contamination (by ELISA method, Boehringer Mannheim). Cells used in the mutation assay were within five subpassages from frozen stock in order to assure karyotypic stability.

B. Culture Condition

HAT-treated CHO-K1 cells were stored in liquid nitrogen. After thawing, cells were maintained as monolayers in McCoy's 5A medium (GIBCO, USA) supplemented with 10% fetal bovine serum and 0.22% sodium bicarbonate (pH 7.0 ~ 7.2) in a humidified incubator at 37 ± 1 °C and 5 ± 1% CO_2 in air.

C. Metabolic Activation System

Aroclor 1254-induced rat liver S9 from Sprague-Dawley rats was used for metabolic activation and was prepared by MOLTOX™, Inc., U.S.A. (Lot no. 1101). S9 containing medium was freshly prepared to contain 100 µl S9/ml reaction mixture with 4

50 mM phosphate buffer (pH 7.4). The S9 reaction mixture was stored on ice until use. For cell treatment, S9 mixture was added to the serum-free medium at the ratio of 1 to 4.

D. Concentration-range Finding Test

The preliminary solubility and toxicity tests were used to establish the optimal concentration levels for the mutagenesis assay. The cytotoxicity assay was consisted of evaluation of test article effect on colony forming efficiency. According to the OECD guideline, the highest concentration level of soluble substance for mutagenesis assay should produce approximate 80-90% cytotoxicity, but not exceed 5 mg/ml or 10 mM.

In the preliminary assay, CHO cells were exposed for 5 hours at 37 ± 1 °C to the test article in the absence of S9 activation and in the presence of S9 activation. After drug exposure, cells were plated at a density of 200 ~ 800 cells/dish and incubated for 7 days without disturbance. According to the test results, 100 μl/ml and 50 μl/ml were set as the top concentrations for the gene mutation assay in the absence and presence of S9 activation, respectively.

E. Test Article Preparation and Test Concentrations

Test article stock solution was prepared in cell culture medium and filtered through 0.2 μm membrane after adjusting pH value to the range of 7.0 ~ 7.2. Cell culture medium was used to make 2-fold serial dilution of test article solutions to desired concentrations for cell treatment. Maximum concentrations of Product code MicrSoy-20 (MS-20) used in different stages were determined as described above. A minimum of four analyzable concentrations has to be obtained for the CHO/HPRT gene mutation analysis. Therefore, additional lower concentrations separated by a factor of 2 were added for cell treatment. Six different concentrations, 3.125, 6.25, 12.5, 25, 50 and 100 μl/ml were used for gene mutation assay in the absence of S9 and 1.5625, 3.125, 6.25, 12.5, 25 and 50 μl/ml for the presence of S9 activation.

F. Controls

1. Positive Control

In the gene mutation assay without S9 activation, methanesulfonic acid, ethyl ester (EMS) [CAS no.62-50-0] (Sigma M0880, USA) at 0.1 μl/ml was used as the positive control. N-nitroso-dimethylamine (DMN) [CAS no.62-75-9] (Sigma N7756, USA) at 100 μg/ml was used for the test with S9 activation. Positive controls were freshly prepared in the culture medium.

2. Solvent Control (Negative Control)

Culture medium was used as the solvent control.

G. Procedures of Gene Mutation Assay (SOP：DCB-DV-TE00232)

1. Preparation of Target Cells

Exponentially growing CHO-K1 cells were seeded in complete McCoy's 5A medium at approximately 1 x 10^6 cells/ 100 mm dish. The dishes were incubated at 37 ± 1 °C and 5 ± 1% CO_2 in a humidified incubator overnight before treatment. Duplicate cultures were used at each concentration of test article and for negative and positive control cultures.

2. Treatment of Target Cells

Treatment was carried out by adding freshly prepared test article or control solutions into cell cultures. For the S9 activation treatment, medium containing S9 reaction mixture at a ratio of 4:1 was used. After incubated for 5 hours at 37 ± 1 °C and 5 ± 1% CO_2 in air, all medium was aspirated. The cells were washed with PBS and incubated in fresh culture medium. After 18-24 hours incubation, the cells were subcultured to assess cytotoxicity and to continue the phenotypic expression period.

3. Estimation of Cytotoxicity

For evaluation of cytotoxicity, the replicate cultures from each treatment condition were subcultured independently in triplicate at approximately 200 ~ 1600 cells/60 mm dish. After 7 days incubation, colonies were fixed with 95% ethanol, stained with 10% Giemsa and counted. Data were recorded in the table DV-TE00232A of SOP: DCB-DV-TE00232.

4. Expression of the Mutant Phenotype

For expression of the mutant phenotype, the replicates from each treatment were subcultured independently at approximately 2 ~ 40 x 10^5 cells/100 mm dish. Subculture as above at 2-3 day intervals was performed for the 7 days expression period.

5. Selection of Phenotype

For selection of the TG-resistant phenotype, cells from each treatment were plated into five dishes at a density of 2 X 10^5 cells/100 mm dish containing 10 μM 6-TG. For cloning efficiency at the time of selection, 200 ~ 400 cells/60 mm dish were plated in triplicate in medium free of 6-TG. After 7 days of incubation, the colonies were fixed, stained and counted for both cloning efficiency and mutant frequency. Both data were recorded in the tables DV-TE00232B and DV-TE00232C of SOP: DCB-DV-TE00232.

H. Data Treatment

The cloning (plating) efficiency of each culture was calculated by dividing the mean colony number by the number of cells plated. The cytotoxic effects of each treatment are expressed relative to the solvent-treated control cultures (relative cloning efficiency). The mutation frequency (MF) for each treatment is calculated by dividing the total number of mutant colonies by the number of cells selected, corrected for the cloning efficiency of cells prior to mutant selection and expressed as TG-resistant mutants per 10^6 clonable cells.

I. Evaluation of Test Results

1. Criteria for a Valid Test

The cloning efficiency of the solvent control must be greater than 50%. The spontaneous mutant frequency in the solvent control must not exceed 25 mutants per 10^6 clonable cells. The positive control must induce a mutation frequency at least 3 times that of the solvent control and must exceed 40 mutants per 10^6 clonable cells. There must be at least four analyzable test article concentrations with mutation frequency data.

2. Data Evaluation

The result interpretation will be based on sound scientific judgment. However, as a guide to evaluate the data, the test article is considered to induce a positive response when fulfills following conditions: (1) There are two consecutive concentrations showing mutant frequencies of ≧ 40 mutants per 10^6 clonable cells, and (2) There is a concentration-related increase in mutant frequencies.

If a single point above 40 mutants per 10^6 clonable cells is observed at the highest concentration, the assay will be considered suspect. If no culture exhibits a mutant frequency of ≧ 40 mutants per 10^6 clonable cells, the test article will be considered negative.

J. Data Retention

All raw data, documentation, records, protocols and final reports generated as a result of this study will be inventoried and archived by the Quality Assurance Unit at DCB's archives located in the Drug Safety Building. The retaining duration of those records will be in accordance with the relevant regulations.

RESULTS AND DISCUSSION

A. Concurrent Cytotoxicity Assay

The HPRT gene mutation assay was analyzed for 5-hour treatment with Product code MicrSoy-20 (MS-20) in non-activated and S9-activated systems. In the non-activated system, cultures were treated with the test article at 6 concentrations, 3.125, 6.25, 12.5, 25, 50 and 100 μl/ml. In S9-activated system, cultures were treated with test article at 6 concentrations, 1.5625, 3.125, 6.25, 12.5, 25 and 50 μl/ml. The cytotoxic effects of the test article (concurrent cytotoxicity) are presented in Tables 1 and 2. The percent of survival was 0% for the non-activated treatment at 100 μl/ml. However, when cells were treated with MS-20 at 50 μl/ml, the survival rate was 16.8% for treatment without S9 activation, and 19.2% for treatment with S9 activation. The result of concurrent cytotoxicity showed that treatment at 50 μl/ml of MS-20 had caused more than 80%

toxicity (cloning efficiency relative to the solvent control) in both the absence and presence of S9 activation.

B. Gene Mutation Assay

After the period of phenotype expression for 7 days, the mutant phenotype was selected by 6-TG-containing medium. The mutation frequencies were calculated based on 10^6 clonable cells (cloning efficiency obtained from Tables 3 and 5) and are presented in Tables 4 and 6. The data of 100 μl/ml concentration treatment were omitted since no viable cells were present. Summary of the results of HPRT gene mutation assay on product code MicrSoy-20 (MS-20) is presented in Table 7. The mutation frequencies of negative controls in both with and without S9 treatments were lower than 25 mutants per 10^6 clonable cells and positive controls gave 97.2 and 125.3 mutants per 10^6 clonable cells, without and with S9 activation, respectively. There were more than 5 concentrations obtained analyzable results. Therefore, the criteria for a valid test were met in this study. None of the treated cultures exhibited mutant frequencies of greater than 40 mutants per 10^6 clonable cells, i.e. no positive response was induced.

CONCLUSION

The criteria for a valid study were met as described in the protocol. The results of the CHO/HPRT gene mutation assay indicate that, under the conditions of this study, Product code MicrSoy-20 (MS-20) did not cause a positive response in both the non-activated and S9-activated systems and was concluded to be negative.

COMMENTS AND/OR PROBLEMS

This study was conducted in compliance with (1) Good Laboratory Practice for Nonclinical Laboratory Studies (21 CFR 58), FDA, U.S.A., 1987; (2) Good Laboratory Practice for Nonclinical Laboratory Studies, Department of Health, R.O.C., 3rd ed., 2000, with the exceptions of test article identification and related analyses.

To the best of our knowledge, there were no deviations from the study protocol that would affect the integrity of this study. No problems were encountered that would adversely affect the study results or interpretation.

REFERENCES

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2. Hsie, A. W., Casciano, D. A., Couch, D. B., Krahn, B. F., O'Neill, J. P. and Whitfield, B. L. (1981) The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals. A report of the Gen-Tox Program. Mutation Research 86: 193-214.
3. Li, A. P., Carver, J. H., Choy, W. N., Hsie, A. W., Gupta, R. S., Loveday, K. S., O'Neill, J. P., Riddle, J. C., Stankowski Jr. L. F., and Yang, L. L. A guide for the performance of the Chinese hamster ovary cell / hypoxanthine-guanine phosphoribosyl transferase gene mutation assay. Mutation Research 189: 135-141.
4. OECD Guideline for the Testing of Chemicals #476 (1997) " *In Vitro* Mammalian Cell Gene Mutation Test".
5. Quality Manual, Drug Development Division, Development Center for Biotechnology, 6th edition, 1999.

Table 1. Concurrent Cytotoxicity Analysis of CHO/HPRT Gene Mutation Assay of Product Code MicrSoy-20 (MS-20) in the Absence of S9

Treatment [a] (μl/ml)	No. of Cell Plated	Colony Forming No.				Plating Efficiency [c] (%)	Mean of Plating Efficiency (%)	% of Survival [d]
		P1	P2	P3 [b]	Mean			
0	200	167	177	190	178.00	89.00	91.58	100.0
0	200	190	192	183	188.33	94.17		
3.125	200	152	182	193	175.67	87.83	83.83	91.5
3.125	200	146	156	177	159.67	79.83		
6.25	200	151	149	149	149.67	74.83	77.92	85.1
6.25	200	153	166	167	162.00	81.00		
12.5	200	156	153	155	154.67	77.33	76.75	83.8
12.5	200	147	145	165	152.33	76.17		
25	200	157	153	154	154.67	77.33	75.17	82.1
25	200	139	154	145	146.00	73.00		
50	400	48	57	53	52.67	13.17	15.42	16.8
50	400	72	70	70	70.67	17.67		
100	1600	0	0	0	0.00	0.00	0.02	0.0
100	1600	0	1	1	0.67	0.04		
P. C. [e]	200	171	166	178	171.67	85.83	85.50	93.4
P. C.	200	177	164	170	170.33	85.17		

[a] Cells were exposed to the test article for 5 hours at 37±1 ℃ in the absence of S9.

[b] P1, P2, P3 = colonies formed in plate 1, plate 2 and plate 3

[c] Plating Efficiency = mean no. of colony formed / no. of cells seeded × 100%

[d] % of survival = Plating efficiency of each treatment / plating efficiency of negative control × 100%

[e] P. C. = Postive control : Methanesulfonic acid, ethyl ester (EMS) at 0.1 μl/ml

Table 2. Concurrent Cytotoxicity Analysis of CHO/HPRT Gene Mutation Assay of Product Code MicrSoy-20 (MS-20) in the Presence of S9

Treatment [a] (μl/ml)	No. of Cell Plated	Colony Forming No.				Plating Efficiency [c] (%)	Mean of Plating Efficiency (%)	% of Survival [d]
		P1	P2	P3 [b]	Mean			
0	200	208	181	185	191.33	95.67	90.92	100.0
0	200	170	176	171	172.33	86.17		
1.5625	200	154	163	159	158.67	79.33	79.50	87.4
1.5625	200	160	163	155	159.33	79.67		
3.125	200	144	173	162	159.67	79.83	73.75	81.1
3.125	200	130	139	137	135.33	67.67		
6.25	200	170	178	169	172.33	86.17	80.00	88.0
6.25	200	152	133	158	147.67	73.83		
12.5	200	127	130	131	129.33	64.67	65.67	72.2
12.5	200	146	128	126	133.33	66.67		
25	200	100	85	99	94.67	47.33	49.42	54.4
25	200	108	93	108	103.00	51.50		
50	400	66	73	85	74.67	18.67	17.46	19.2
50	400	64	61	70	65.00	16.25		
P. C. [e]	200	106	114	111	110.33	55.17	50.92	56.0
P. C.	200	107	90	83	93.33	46.67		

[a] Cells were exposed to the test article for 5 hours at 37±1 ℃ in the presence of S9.

[b] P1, P2, P3 = colonies formed in plate 1, plate 2 and plate 3

[c] Plating Efficiency = mean no. of colony formed / no. of cells seeded × 100%

[d] % of survival = Plating efficiency of each treatment / plating efficiency of negative control × 100%

[e] P. C. = Postive control : N-nitroso-dimethylamine (DMN) at 100 μg/ml

Table 3. Effects of Product Code MicrSoy-20 (MS-20) on Cloning Efficiency in CHO/HPRT Gene Mutation Assay in the Absence of S9

Treatment [a] (μl/ml)	No. of Cell Plated	Colony Forming No.				Cloning Efficiency [c] (%)
		P1	P2	P3 [b]	Mean	
0	200	201	218	207	208.67	104.3
0	200	169	179	187	178.33	89.2
3.125	200	186	176	172	178.00	89.0
3.125	200	202	171	179	184.00	92.0
6.25	200	173	181	181	178.33	89.2
6.25	200	169	193	190	184.00	92.0
12.5	200	187	180	172	179.67	89.8
12.5	200	175	147	183	168.33	84.2
25	200	150	163	166	159.67	79.8
25	200	169	172	128	156.33	78.2
50	400	322	339	261	307.33	76.8
50	400	344	300	357	333.67	83.4
P. C. [d]	200	138	153	161	150.67	75.3
P. C.	200	149	149	145	147.67	73.8

[a] Cells were exposed to the test article for 5 hours at 37±1 ℃ in the absence of S9.

[b] P1, P2, P3 = colonies formed in plate 1, plate 2 and plate 3

[c] Cloning Efficiency = mean no. of colony formed / no. of cells seeded × 100%

[d] P. C. = Postive control : Methanesulfonic acid, ethyl ester (EMS) at 0.1 μl/ml

Table 4. Effects of Product Code MicrSoy-20 (MS-20) on the Induction of Mutation Frequency in CHO/HPRT Gene Mutation Assay in the Absence of S9

Treatment [a] (μl/ml)	Colony Forming No.					Total Mutant Colonies	Cloning Efficiency [c] (%)	Mutants / 10^6 Clonable Cells [d]
	P1	P2	P3	P4	P5 [b]			
0	0	0	0	0	0	0	104.3	0.0
0	0	0	0	0	0	0	89.2	0.0
3.125	6	4	3	5	2	20	89.0	22.5
3.125	0	0	0	0	0	0	92.0	0.0
6.25	4	0	0	2	0	6	89.2	6.7
6.25	0	1	0	2	1	4	92.0	4.3
12.5	0	0	0	1	0	1	89.8	1.1
12.5	0	2	0	4	0	6	84.2	7.1
25	2	1	3	0	0	6	79.8	7.5
25	1	0	1	4	3	9	78.2	11.5
50	1	1	0	0	0	2	76.8	2.6
50	2	4	2	1	1	10	83.4	12.0
P. C. [e]	15	14	14	17	14	74	75.3	98.2
P. C.	15	16	9	16	15	71	73.8	96.2

[a] Cells were exposed to the test article for 5 hours at 37±1 ℃ in the absence of S9.

[b] P1, P2, P3, P4, P5 = colonies formed in plate 1, plate 2, plate 3, plate 4 and plate 5

[c] Cloning Efficiency = mean no. of colony formed / no. of cells seeded × 100%

[d] Mutants / 10^6 clonable cells = Total mutant colonies / (Number selection dishes × Cloning efficiency × 2 × 10^5 cells) × 10^6

[e] P. C. = Postive control : Methanesulfonic acid, ethyl ester (EMS) at 0.1 μl/ml

Table 5. Effects of Product Code MicrSoy-20 (MS-20) on Cloning Efficiency in CHO/HPRT Gene Mutation Assay in the Presence of S9

Treatment [a] (μl/ml)	No. of Cell Plated	Colony Forming No.				Cloning Efficiency [c] (%)
		P1	P2	P3 [b]	Mean	
0	200	167	172	179	172.67	86.3
0	200	194	176	144	171.33	85.7
1.5625	200	142	164	152	152.67	76.3
1.5625	200	154	144	141	146.33	73.2
3.125	200	173	159	163	165.00	82.5
3.125	200	115	156	159	143.33	71.7
6.25	200	126	131	138	131.67	65.8
6.25	200	145	130	144	139.67	69.8
12.5	200	140	145	142	142.33	71.2
12.5	200	143	150	148	147.00	73.5
25	200	124	124	125	124.33	62.2
25	200	150	161	142	151.00	75.5
50	400	206	193	216	205.00	51.3
50	400	276	245	234	251.67	62.9
P. C. [d]	200	118	116	106	113.33	56.7
P. C.	200	119	131	143	131.00	65.5

[a] Cells were exposed to the test article for 5 hours at 37±1 ℃ in the presence of S9.

[b] P1, P2, P3 = colonies formed in plate 1, plate 2 and plate 3

[c] Cloning Efficiency = mean no. of colony formed / no. of cells seeded × 100%

[d] P. C. = Postive control : N-nitroso-dimethylamine (DMN) at 100 μg/ml

Table 6. Effects of Product Code MicrSoy-20 (MS-20) on the Induction of Mutation Frequency in CHO/HPRT Gene Mutation Assay in the Presence of S9

Treatment [a] (μl/ml)	Colony Forming No.					Total Mutant Colonies	Cloning Efficiency [c] (%)	Mutants / 10^6 Clonable Cells [d]
	P1	P2	P3	P4	P5 [b]			
0	0	2	1	0	6	9	86.3	10.4
0	0	3	0	1	0	4	85.7	4.7
1.5625	5	2	3	2	1	13	76.3	17.0
1.5625	0	0	1	1	2	4	73.2	5.5
3.125	0	0	0	1	5	6	82.5	7.3
3.125	0	0	0	0	0	0	71.7	0.0
6.25	1	1	2	0	2	6	65.8	9.1
6.25	1	4	2	0	2	9	69.8	12.9
12.5	2	1	5	3	2	13	71.2	18.3
12.5	8	0	2	0	0	10	73.5	13.6
25	1	4	9	7	0	21	62.2	33.8
25	8	2	2	0	2	14	75.5	18.5
50	0	0	0	0	4	4	51.3	7.8
50	4	4	4	0	4	16	62.9	25.4
P. C. [e]	11	13	6	16	12	58	56.7	102.4
P. C.	17	26	15	24	15	97	65.5	148.1

[a] Cells were exposed to the test article for 5 hours at 37±1 °C in the presence of S9.

[b] P1, P2, P3, P4, P5 = colonies formed in plate 1, plate 2, plate 3, plate 4 and plate 5

[c] Cloning Efficiency = mean no. of colony formed / no. of cells seeded × 100%

[d] Mutants / 10^6 clonable cells = Total mutant colonies / (Number selection dishes × Cloning efficiency × 2 × 10^5 cells) × 10^6

[e] P. C. = Postive control : N-nitroso-dimethylamine (DMN) at 100 μg/ml

Table 7. Summary of the Results of CHO/HPRT Gene Mutation Assay on Product code MicrSoy-20 (MS-20)

Treatment [a] (μl/ml)	S9	Cytotoxicity (%) [b]	Mutation Frequency [c] (per 10^6 clonable cells)
0	-	0	0
3.125	-	8.5	11.3
6.25	-	14.9	5.5
12.5	-	16.2	4.1
25	-	17.9	9.5
50	-	83.2	7.3
100	-	100	-
P. C. [d]	-	6.6	97.2
0	+	0	7.6
1.5625	+	12.6	11.3
3.125	+	18.9	3.7
6.25	+	12.0	11
12.5	+	27.8	16.0
25	+	45.6	26.2
50	+	80.8	16.6
P. C. [d]	+	44.0	125.3

[a] Cells were exposed to the test article for 5 hours at 37±1 ℃.

[b] Cytotoxicity (%) = 1 – (% of Survival). Survival data were from Tables 1 and 2.

[c] Mutation Frequency: Average of duplicate cultures from Tables 4 and 6.

[d] P. C.: Positive controls in treatments without and with S9 were described in Tables 4 and 6, respectively.

Appendix A

- Test Article Information Sheet

DB DEVELOPMENT CENTER FOR BIOTECHNOLOGY

Test Article Information Sheet DV00199-1

DV-QA00033E

Sponsor : MICROBIO Co., Ltd.

Address : No.81 Gauyang N. Rd., Lung tan Shiang, Tao Yuan, Taiwan

Telephone : 886-3-4710888 Fax : 886-3-4710288

Delivery Date : 10 / 05 / 2001 (MM / DD / YY)

Category : ☐Health Food ☑Herb Medicine ☐Drugs ☐Cleanser ☐Medical Devices ☐Cosmetics ☐Pesticides ☐Others : _____

1. Sample Name : Product code MicrSov-20(MS-20)

2. a. Ingredients :

MS-20 is a Chinese medicine. The components are very complicated. Until now, its effective components are still unable to determine.

b. Purity : _____

3. Batch / Lot No. : 20010209

4. Physical Appearance :

a. ☐Powder ☑Liquid ☐Others : _____

b. Odor : ☐No ☑Yes : Prune Juice

c. Color : Dark-brown

5. How Supplied (Amount / Pack) : 30 ml/Bottle

6. Amount Supplied : 2 Bottle

DB DEVELOPMENT CENTER FOR BIOTECHNOLOGY

Test Article Information Sheet DV00199-1

7. Solubility (Approx. ____ g/L)
   H_2O Souble · DMSO _____ · Other Solvents _____

8. Storage
   a. Storage Temperature : ☑Room Temperature ☐Refrigeration ☐Frozen
   b. Other Environment Condition : ☐Desiccation ☑Protect from Light
      ☐Others : _____
   c. Expiration Date : 02/ 09/ 2004 (MM / DD / YY)

9. Treatment of Residual Samples
   ☑ Retrieved by the Sponsor
   ☐ Managed by DCB with Extra Fees
   ☐ Disposed by DCB with Waste Disposal Method Provided :

10. **Handling Precautions** and Others

Directions: To drink 1~5c.c. daily by dilution with 100c.c. water before breakfast. It's not suggested to drink water in 10 minutes after MS-20. After 10 minutes later, we suggest you to drink water as usual. Before dilution, the product can be stored at room temperature after opening, but please use the product immediately after dilution.

Undiluted product has a high acidity of pH around 3.8

MS-20 has two packages which are 180ml/Bottle and 30ml/Bottle

Product Chemist : _____ _____ (Signature)
(MM / DD YY)

Sponsor Representative : _____ _____ (Signature)
(MM / DD YY)

Appendix B

- Protocol

HPRT GENE MUTATION ASSAY
PRODUCT CODE MICRSOY-20 (MS-20)

PROTOCOL

DEVELOPMENT CENTER FOR BIOTECHNOLOGY
DRUG DEVELOPMENT DIVISION

Signature Page

Study Director:

Shwu-Fer Lee-Chen Nov. 14, 2001

Dr. Shwu-Fer Lee-Chen, Ph.D.

Investigators:

Lu-Jane Lin, B.S.

Chun-Han Shih, M.S.

Quality Assurance Officer:

Wen-Jing Chen Nov. 14, 2001

Wen-Jing Chen, M.S.

Facility Manager:

Jiuan Judy Liu Nov. 14, 2001

Dr. Jiuan Judy Liu, DVM, Ph.D

Sponsor's Representative:

Nov, 16, 2001

William Lu

Jason Hu for William Lu

HPRT GENE MUTATION ASSAY – PRODUCT CODE MICRSOY-20 (MS-20)

I. PURPOSE

The objective of HPRT gene mutation assay is to assess the mutagenic potential of a test article based on quantitation of forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus of Chinese hamster ovary (CHO) cells.

II. TESTING FACILITY

A. Name: Development Center for Biotechnology, Genetic Toxicology Laboratory

B. Address: 103, Lane 169, Kang-Ning St., Hsi-Chih City, Taipei County 221, Taiwan, R.O.C.

III. SPONSOR

A. Name: MICROBIO Co., Ltd.

B. Address: No. 81, Gauyang N. Road, Lung Tan Shiang, Tao Yuan, Taiwan.

C. Representative: William Lu

IV. TEST ARTICLE (It was supplied by the sponsor prior to study initiation)

A. Name/Identification: Product code MicrSoy-20 (MS-20)

B. Receiving Date: Oct. 05, 2001

C. Batch/Lot Number: 20010209

D. DCB Code: DV00199-1

E. Ingredients: MS-20 is a Chinese medicine. The components are very complicated. Until now, its effective components are still unable to determine.

F. Storage Conditions: Room temperature and protect from light

G. Expiration Date: Feb. 09, 2004

H. Physical Appearance: Dark-brown liquid with prune juice odor

Statement:

The test article is a proprietary product of the sponsor, therefore the sponsor will be responsible for the requirements listed under "Test Article" of the GLP regulation (21CFR § 58.105, FDA).

V. TEST SCHEDULE

A. Proposed First Date of Target Cells Seeding: Nov. 19, 2001
B. Proposed First Date of Test Article Treatment: Nov. 20, 2001
C. Proposed Date of Result Observation: Dec. 11, 2001

VI. TEST SYSTEM

A. Cells: Chinese hamster ovary cells (CHO-K1)
B. Source: American Type Culture Collection (ATCC, repository number CCL-61), Rockville, MD, USA.
C. Modal Chromosome Number: 20
D. Population Doubling Time: About 12~14 hours
E. Mycoplasma: Negative
F. Cell Culture Condition:

CHO-K1 cells were cleansed in medium supplemented with HAT (hypoxanthine, aminopterin and thymidine) then frozen in liquid nitrogen. After thawing, cells will be maintained as monolayers in McCoy's 5A medium (GIBCO, USA) supplemented with 10% (v/v) fetal bovine serum and 0.22% sodium bicarbonate (pH 7.0~7.2) in a humidified incubator at 37± 1 °C and 5± 1% CO_2 in air. Cells used for mutation assay will not exceed five subpassages from frozen stock.

G. Reason for System Selection:

The CHO/HPRT assay was designed to select for mutant cells that have become resistant to purine analogues such as 6-thioguanine (6-TG) as a result of mutation at the X-chromosome-linked HPRT locus. This system has been demonstrated to be sensitive to the mutagenic action of a variety of chemicals (Hsie et al., 1981) and is suggested to be one of *in vitro* mammalian cell gene mutation assays by OECD guideline (1997).

VII. EXPERIMENTAL DESIGN

A. Experimental Design

The assay will be performed by exposing CHO-K1 cells for 5 hours to a minimum of 5 concentrations of test article as well as positive and negative controls in the presence and absence of S9 metabolic activation. After 7-9 day expression period, the treated cells will be cultured in the presence of 6-TG for selection of mutant colonies. The mutagenic potential of a test article will be determined by its

ability to induce a concentration-response increase in mutant frequency within clonable cells.

B. Test Article Preparation and Concentration-Range Finding Test

The solution of test article was prepared according to the Sponsor's information. Sample of MicrSoy-20 (MS-20) was diluted in culture medium and filtered through 0.2 μm membrane after adjusting pH value to the range of 7.0 ~ 7.2. A preliminary cytotoxicity assay with colony forming efficiency was performed to determine the optimal concentrations for HPRT gene mutation assay.

According to the regulatory guidelines, the highest concentration of mammalian gene mutation assay should produce approximately 80-90% cytotoxicity and the maximum concentration for soluble non-toxic substance is 5 mg/ml or 10 mM.

The results of preliminary test showed that the test article gave 79.5% and 88.9% of cytotoxicity when cells treated at 50 μl/ml for 5 hours without and with S9 activation, respectively. Therefore, 100 μl/ml will be set as the top concentration in the absence of S9 activation, 50 μl/ml as the top concentration in the presence of S9 activation for the gene mutation assay. Test article will be prepared in the culture medium for cell treatment.

C. Exposure Concentrations and Duration

Based on the results of preliminary cytotoxicity assay, top concentrations and additional 5 concentration levels with a dilution factor of 2 will be conducted to obtain a minimum of four analyzable concentrations in the gene mutation assay. Test concentrations are listed below: in the absence of S9 mix, 3.125, 6.25, 12.5, 25, 50 and 100 μl/ml; in the presence of S9 mix, 1.5625, 3.125, 6.25, 12.5, 25 and 50 μl/ml. Cell cultures will be treated for 5 hours with control and test articles both in the presence and absence of S9 activation.

D. Controls

1. Positive Control

In the gene mutation assay without S9 activation, methanesulfonic acid ethyl ester (EMS) [CAS no. 62-50-0] (Sigma M0880, USA) at 0.1 ~ 0.2 μl/ml will be used as the positive control. N-nitroso-dimethylamine (DMN) [CAS no. 62-75-9] (Sigma N7756, USA) at 100 ~ 200 μg/ml will be used for the test with S9 activation. Positive controls will be freshly prepared in the culture medium.

2. Negative Control

Culture medium will be used as the negative control.

E. Metabolic Activation

Aroclor 1254-induced rat liver S9 from Sprague-Dawley rats will be used for metabolic activation and was prepared by MOLTOX™, Inc., U.S.A. (Lot no. 1101). S9 containing medium will be freshly prepared to contain 100 µl S9/ml reaction mixture with 4 mM NADP, 5 mM G-6-P, 10 mM $MgCl_2 \cdot 6H_2O$, 30 mM KCl, 10 mM $CaCl_2 \cdot H_2O$ and 50 mM phosphate buffer (pH 7.4). The S9 reaction mixture will be kept on ice until use. For cell treatment, S9 mixture will be added to the serum-free medium at the ratio of 1 to 4.

F. Procedures of Gene Mutation Assay (SOP：DCB-DV-TE00232)

1. Preparation of Target Cells

Exponentially growing CHO-K1 cells will be seeded in complete McCoy's 5A medium at approximately 1 x 10^6 cells/ 100 mm dish. The cells will be incubated overnight before treatment. Duplicate cultures will be used at each concentration of test article and for negative and positive control cultures.

2. Treatment of Target Cells

Treatment will be carried out by adding freshly prepared test article or control solutions into cell cultures. After incubated for 5 hours at 37± 1 °C and 5 ± 1% CO_2 in air, all medium will be aspirated. The cells will be washed with PBS and incubated in fresh culture medium. After 18-24 hours incubation, the cells will be subcultured to assess cytotoxicity and to continue the phenotypic expression period.

3. Estimation of Cytotoxicity

For evaluation of cytotoxicity, the replicate cultures from each treatment condition will be subcultured independently in triplicate at a density of 100 ~ 200 cells/60 mm dish. After 7-10 days incubation, colonies will be fixed with 95% ethanol, stained with 10% Giemsa and counted. Cytotoxicity will be expressed relative to the negative control cultures.

4. Expression of the Mutant Phenotype

For expression of the mutant phenotype, the replicates from each treatment will be subcultured independently at approximately 5 X 10^5 cells/100 mm dish. Subculture as above at 2-3 day intervals will be performed for the 7-9 day expression period.

5. Selection of Phenotype

For selection of the TG-resistant phenotype, cells from each treatment will be plated into five dishes at a density of 2 X 10^5 cells/100 mm dish containing 10 µM 6-TG. For cloning efficiency at the time of selection, 100 ~ 200 cells/60 mm

dish will be plated in triplicate in medium free of 6-TG. After 7-10 days of incubation, the colonies will be fixed, stained and counted for both cloning efficiency and mutant frequency.

VIII. CRITERIA FOR A VALID TEST

A. Negative Controls

The cloning efficiency of the negative control must be greater than 50%. The spontaneous mutant frequency in the negative control must not exceed 25 mutants per 10^6 clonable cells.

B. Positive Controls

The positive control must induce a mutation frequency at least 3 times that of the negative control and must exceed 40 mutants per 10^6 clonable cells.

C. Test Article Treated Cultures

A minimum of four analyzable concentrations with mutation frequency data should be obtained.

IX. STATISTICAL ANALYSIS

A. Data Treatment

The cytotoxic effects of each treatment will be expressed relative to the negative control cultures (relative cloning efficiency). The mutation frequency (MF) for each treatment will be calculated by dividing the total number of mutant colonies by the number of cells selected, corrected for the cloning efficiency of cells prior to mutant selection and expressed as TG-resistant mutants per 10^6 clonable cells. Mutant frequencies generated from concentrations giving $\leq$ 10% relative survival will be presented in the data but will be not considered as valid data points.

B. Data Evaluation

The result interpretation will be based on sound scientific judgement. However, as a guide to evaluate the data, the test article is considered to induce a positive response when fulfills following conditions.

1. There are two consecutive concentrations showing mutant frequencies of $\geqq$ 40 mutants per 10^6 clonable cells.
2. There is a concentration-related increase in mutant frequencies.

If a single point above 40 mutants per 10^6 clonable cells is observed at the highest concentration, the assay will be considered suspect. If no culture exhibits a

mutant frequency of ≧ 40 mutants per 10^6 clonable cells, the test article will be considered to induce a negative response.

X. RECORDS RETENTION

All raw data, documentation, records, protocols and final reports generated as a result of this study will be inventoried and archived by the Quality Assurance Unit at DCB's archives located in Drug Safety Building. The retention duration of those records will be in accordance with the relevant regulations.

XI. REGULATORY REQUIREMENTS

Except the parts of "test article" requirement, this study will be performed in compliance with (1) Good Laboratory Practice for Nonclinical Laboratory Studies (21 CFR 58), FDA, U.S.A., 1987; (2) Good Laboratory Practice for Nonclinical Laboratory Studies, Department of Health, R.O.C., 3rd ed., 2000; (3) General Requirements for the Competence of Calibration and Testing Laboratories (ISO/IEC Guide 25), ISO/IEC, 3rd ed., 1990; (4) Specific Criteria for Biological Testing, Chinese National Laboratories Accreditation, R.O.C., 2nd ed., 2000.

XII. REFERENCES

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